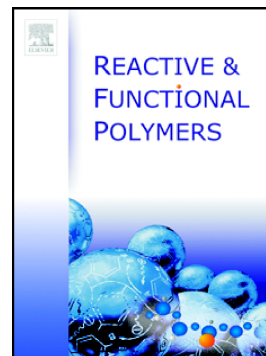# Journal Pre-proof

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Biological properties of direct grafting by ultraviolet irradiation of vinyl benzyl phosphonic acid onto titanium surfaces

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Abstract: Titanium (Ti) and its alloys are the most effective metals for structural implantable device applications. However, Ti-based materials are passively integrated into the bone, resulting in a purely mechanical attachment. Consequently, the loss of osseointegration often leads to implant failure. Therefore, enhancing bone formation surrounding the implant is primordial. In previous investigations conducted in our laboratory, grafting bioactive polymers with sulfonate groups, such as poly(sodium styrene sulfonate) (polyNaSS), was demonstrated to increase the adherence and differentiation of osteoblast cells. In this context, this contribution proposes to functionalize Ti with a phosphonic acid-based polymer, poly(vinyl benzyl phosphonic acid) (poly(VBP)). A two-step UV-initiated grafting polymerization was developed to covalently graft VBP into Ti surfaces. The surfaces were characterized using colorimetry, Fourier-transformed infrared spectra recorded in an attenuated total reflection (FTIR-ATR), X-ray photoelectron spectroscopy (XPS) techniques, and water contact angle (WCA) measurements. The Ti substrates were evaluated for cell viability, spreading, alkaline phosphatase activity, and calcium formation using MC3T3-E1 osteoblast cells. The interaction of Ti grafted samples with osteoblast cells was significantly improved as well as the cell/surface interaction. Together, these findings demonstrated that poly(VBP) grafted on Ti surfaces improved osteoblasts' early cell adhesion and spreading activities, crucial for osseointegration applications.
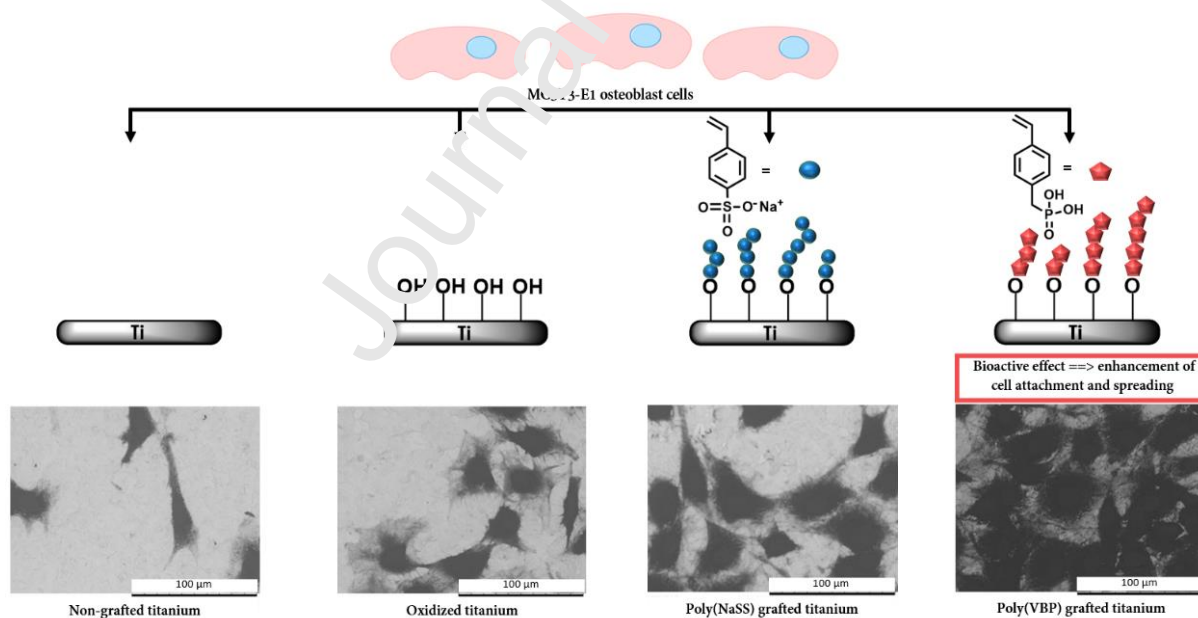
Graphical abstract

1. Introduction

Titanium (Ti) and its alloys, employed in the biomedical field since the early 1950s [1], have demonstrated the success of their mechanically-compatible properties as biomedical devices. However, despite their biocompatibility, titanium implants are not optimally osseointegrated, and an inflammatory process resulting in fibrous capsule development can ensue. As a result, implants are encased by fibrous tissue, isolating them from the bone tissue and causing implant loosening [2,3].

The success of implant surgery is primarily based on the complex osseointegration mechanisms involving both mechanical and biochemical aspects occurring at the contact between the bone tissue and the implant surface. The effectiveness of biomaterial placement is closely linked to the osseointegration phenomenon that occurs when the bone tissue adjusts to the presence of the implant through the bone remodeling mechanisms. Therefore, an exemplary bone/implant interface would consist of bone merged with the implant surface without a fibrous connective tissue layer in between.

To improve the bone uptake of the implant, researchers have developed different strategies. Modifications of the morphological surface properties (roughness, porosity) using various methods, essentially mechanical treatments, allow an essential range of roughness and textures that promote bone bonding [4]. Other approaches have been used to improve osseointegration, including physical modifications (thermal treatment, plasma treatment) [5-7], chemical modifications (silane treatments, protein coating) [8], and electrochemical treatments (anodic oxidation) [9,10]. Moreover, to improve osteoblast responses and bone-implant integration, chemical modifications were made to directly functionalize Ti surfaces with various chemical groups, like hydroxyl [11], carboxyl [12], sulfonic groups [13], and phosphate groups [14]. As a result of our prior research, polymers bearing ionic chemical functions have been found to modulate cell attachment and spreading [15,16]. The arrangement of these ionic groups throughout the polymer chains affords effective sites that operate with extracellular proteins vital in cell response, such as fibronectin [17]. Despite the effectiveness of the approaches described above, numerous limitations remain, such as poor coating-to-substrate adhesion and sophisticated and time-consuming chemical processes. Grafting polymerization induced by ultraviolet (UV) light is an easy, quick, and low-cost approach for introducing desired functional groups to the surfaces of materials without changing their bulk characteristics. Our laboratory has developed a direct UV grafting process of a bioactive monomer such as sodium styrene sulfonate (NaSS) [18]. By activating the Ti surface with hydroxyl, it is possible to generate active radicals under UV irradiation. The polymerization of functional monomers is induced by radical species, resulting in bioactive groups on the surface of Ti.

One interesting molecule to graft is a phosphonic acid-based monomer. Studies have shown that titanium and polymers functionalized with phosphate groups ($-PO_3H_2$) exhibit enhanced bioactivity due to their bone-like apatite-induced ability [19-21]. In recent years, vinyl phosphonic acid was used as a coating to manufacture bone graft replacements [22,23] and functionalize bone scaffolds [24] to stimulate regenerating bones. These findings suggested the beneficial influence of phosphonate groups on implant osseointegration.

The current work describes a method for improving bone-implant integration by functionalizing Ti with a phosphonic-acid-based monomer: vinyl benzyl phosphonate (VBP). This study used a two-step UV-induced graft polymerization of VBP to functionalize Ti surfaces with phosphonate groups. Ti oxidation produces peroxides on the surface, which generate radicals when exposed to UV light, initiating the monomer VBP polymerization. Various factors for optimizing poly(VBP) grafting on titanium substrates were investigated. The surface grafting effect was chemically characterized. Furthermore, *in vitro* investigations on MC3T3-E1 osteoblast cells were assessed to evaluate how the grafted Ti surfaces influenced cell adherence, spreading, and differentiation.

2. Materials and Methods

2.1. Materials

Grade 2, commercially pure Ti disks of 10 mm diameter and ≈ 1 mm thickness purchased from Goodfellow (supplier set in Lille, France) were used as raw surfaces. Both sides of the Ti were polished with 500 and 1200 grit SiC papers.

(4-Vinylbenzyl)phosphonic acid (VBP) was supplied by Specific Polymers (supplier set in Montpellier France) and stored at 4°C. The monomer was used without further purification, as it is provided without polymerization inhibitor.

Recrystallization in a combination of ethanol/water (Carlo Erba) (10: 90 v/v) overnight at 70°C was used to purify sodium styrene sulfonate (NaSS, Sigma). The product was then dried overnight at 50°C under air pressure and kept at 4°C as previously described [18,25,26].

2,2'-azobis(2-methylpropionitrile) (AIBN) was refined by the same recrystallization described above for 1h at 30°C. The purified AIBN was vacuum-dried at 30°C and stored at 4°C.

The solvents used for the cleaning treatment, activation procedure and functionalization of titanium surfaces were used without further purification.

2.2. Methods

2.2.1. Cleaning treatment of titanium substrates

The surfaces were ultrasonically cleaned for 15 minutes following the polishing procedure with different solvents: acetone, cyclohexane, isopropanol, and distilled water (dH_2O). After that, Ti surfaces were then stripped for 1 min with stirring in Kroll's reagent (2% hydrofluoric acid (Sigma) ; 10% HNO_3 (Acros organics) and 88% dH_2O), followed by 15 min of ultrasonic washing in five successive distilled water baths. Ti substrates were then dried in an oven at 50°C.

2.2.2. Activation procedure of titanium substrates

Briefly, Ti substrates were chemically oxidized by immersion with an acidic solution (H_2SO_4/H_2O_2 50:50 v/v, Sigma) to activate the surfaces with hydroxide and peroxide groups. Then, the Ti surfaces were rinsed with dH_2O to remove any traces of the Piranha solution.

2.2.3. Grafting procedure of VBP onto titanium surfaces

A 1 M VBP solution in dimethyl sulfoxide (DMSO) was poured into a flask with a circular bottom that contains 2% of 2,2'-azobis(2-methylpropionitrile) (AIBN). After degassing the solution with argon for 30 minutes, the oxidized Ti substrates were quickly added to the solution. The Ti surfaces immersed in the VBP/DMSO solution containing 2% AIBN were then irradiated with UV radiation (365 nm, 160 mW/cm^2 with a power of 38%) at ambient temperature for 2h while stirring (**Fig. 1**). The grafted surfaces were then washed with a mixture of DMSO/methanol (50:50 v/v, Sigma). Then the surfaces were washed with methanol for 48h to remove nonreacted monomers and non-grafted homopolymers. Finally, the poly(VBP) grafted Ti surfaces were dried overnight at 37°C.

Under normal temperature and pressure conditions, UV sources for the grafting technique come from an LED lamp (Omnicure) with a produced power ranging from 200 W to 500 W at 365 nm.
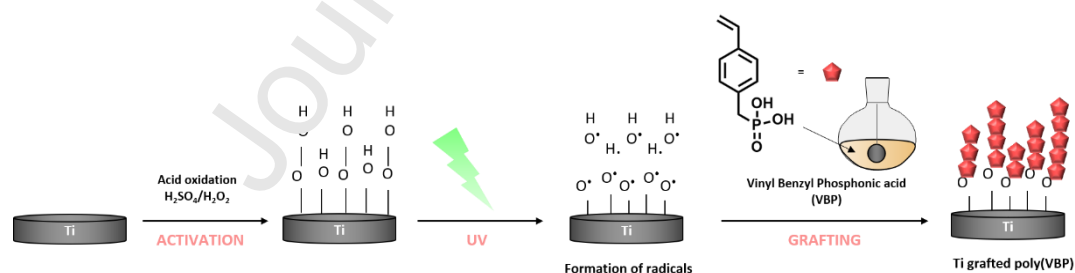

Fig. 1. Grafting mechanism of poly(VBP) on titanium surface

2.3. Surface characterization

Fourier-transformed infrared (FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS), water contact angle measurements (WCA), and the toluidine blue (TB) colorimetric technique were used to assess the existence of poly(VBP) on grafted Ti surfaces.

In attenuated total reflectance (ATR) mode, FTIR spectra were collected between 4000 and 600 cm^{-1}. Ti disks were pushed equally against a diamond crystal (4000-500 cm^{-1}) (512 scans). After that, each spectrum was fitted and examined.

Under ultra-high vacuum circumstances (≤10-10 Torr), photoelectrons emission was studied at a 90° take-off angle. The chemical contents of the surface were analyzed using XPS (from the Omicron Argus spectrometer in Taunusstein, Germany) with a monochromated Alkα radiation source (hν = 1486.6 eV) and a 300 W electron beam. The survey spectrum was captured with a pass energy of 100 eV and the high-resolution

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areas with 20 eV. The C1s binding energy of aliphatic carbon atoms, 284.8 eV, was used to calibrate the binding energies. Casa XPS v.2.5.15 software (Casa Software Ldt, UK) was used to profile high-resolution spectra, and the resultant peaks regions were utilized to compute the elemental composition.

The wettability of the samples was measured after each surface treatment phase by measuring WCA with one drop (2 μl) of dH2O using a Kruss DAS10 goniometer at ambient temperature. Six samples from each group were examined, with three measurements made on each to determine the average contact angle 20 seconds after the water drop made contact with the surface. The contact angle was evaluated using the DSA drop shape analysis tool after photos of the droplet were obtained.

The TB colorimetric technique was used to evaluate the quantity of poly(VBP) grafted on Ti surfaces, following the methodology established by Helary et al [25]. To facilitate the complexation of TB with the PO_3^{2-} groups from the polymer, Ti surfaces were submerged in a TB aqueous solution (5×10^{-4} M, pH = 10) at 30°C for 6 hours. To remove the non-complexed dye, the surfaces were washed with 5×10^{-3} M sodium hydroxide in dH2O. A combination of acetic acid/dH2O (50/50 v/V Sigma) was used to de-complex the disks for 24 hours. Finally, the concentration of de-complexed TB was determined using a Perkin-Elmer Lambda 25 spectrometer using visible spectroscopy at 633 nm. Three Ti samples of each condition (non-grafted, oxidized, poly(NaSS) grafted, and poly(VBP) grafted) were used per analysis.

2.4. Preparation of titanium disks for biological assays

All substrates (ungrafted, oxidized, poly(NaSS), and poly(VBP) grafted) were cleaned and sterilized before interaction with the osteoblast cells. Ti disks were washed three times for three hours in 1.5 M sodium chloride (NaCl, Fisher), 0.15 M sodium chloride, pure water, and phosphate-buffered saline solution, respectively (PBS, Gibco). For 15 min, each side of the Ti substrates was sterilized using an ultraviolet germicidal lamp (UV, 30 W, 254 nm). The samples were kept overnight on well-plates with Gibco's Dulbecco's Modified Eagle's Medium (DMEM). Finally, the samples were put in wells with enriched DMEM medium (1% penicillin, 1% glutamine, 10% fetal bovine serum) and kept until the experiments began.

2.5. Osteoblastic cell culture

All tests were carried out using MC3T3-E1 cells procured from the American Type Culture Collection (ATCC). The cells were cultivation was based on the protocol established by Felgueiras et al [17]. The cell culture media were replaced twice a week. All investigations employed just early passage (P2-P6) cells. To separate the cells, trypsin-EDTA (Gibco) was utilized. All studies used a cell loading dose of 1×10^5 cells/ml, with 1 ml of cell suspension introduced onto each Ti surface kept in a 24-well tissue culture polystyrene plate. Analysis of variances (ANOVA) was used to analyze quantitative data.

2.5.2. Cell viability

A MTT (3- (4,5-dimethyl thiazolyl-2) – 2,5- diphenyltetrazolium bromide) test was used to determine the vitality of the cells. The Ti substrates were first seeded for 24 hours. The samples were then transferred to a fresh 24-well plate and washed twice with 1 ml of PBS per well after the culture. The wells were then filled with 50 μl of 5 mg/ml MTT in a DMEM medium without phenol red. The samples were incubated at 37°C with 5% CO_2 for 4 hours. After incubation, all of the solutions were withdrawn from the wells, and the formazan crystals were dissolved in 1 ml of DMSO for 10 min during orbital stirring. A UV-Vis reader was used to measure the solution's absorbance (PerkinElmer lambda 25).

2.5.3. Cellular spreading and morphology

Environmental scanning electron microscopy pictures (ESEM – Hitachi TM3000) were used to analyze the cellular morphology on ungrafted, oxidized, poly(NaSS), and poly(VBP) grafted surfaces 1h, 4h, and 24h after incubation.

The Ti surfaces were first cleaned in PBS after being withdrawn from the cultures. The cells were then fixed for 30 minutes at 4°C with 4% formaldehyde (Sigma) in PBS.

2.5.4. Differentiation: Alkaline phosphatase activity

ALP is a precursor to the development of osteoblasts. After 7 and 14 days of culture, its activity was measured after p-nitrophenyl phosphate was converted to p-nitrophenol at 37°C. The enzyme was isolated from cell's membrane for 1 hour under agitation using Triton x 100. The cellular suspension was coupled with 20 mM

p-nitrophenyl phosphate substrate in a 2-amino-2-methyl-propanol buffer in the same quantities and kept at 37°C for 30 minutes. A UV-vis spectrometer set to 405 nm was used to measure the amount of p-nitrophenol generated (Perkin-Elmer). The enzyme activity was normalized to the protein mass and expressed in nmol of p-nitrophenol generated per minute (expressed in mg). A commercially available colorimetric assay was used to determine the amount of ALP in the sample (Bio-Rad protein kit assay).

2.5.5. Extracellular matrix mineralization: Calcium production

After 3 and 4 weeks of culture, the mineralization of calcium deposits was assessed. The Ti surfaces and culture medium were removed, washed two times in PBS, and transferred to a fresh well containing 1 ml of 15% (w/v) trichloroacetic acid solution to dissolve calcium-phosphate crystals. To test the calcium concentration, 10 µl of the supernatant was added to 1 ml of arsenazo-III (0.2 mM in PBS) containing calcium reagent after 1 h of continuous gentle shaking. The calcium concentration was estimated by comparing absorbance at 650 nm to a standard linear curve of $CaCl_2$ in trichloroacetic acid (15% w/v) from 50 to 1000 µg.ml^{-1}.

2.6. Statistical analysis

Each experiment was carried out three times and yielded the same finding. The data were provided as mean standard deviation with n = 9. The standard deviations are indicated by the error bars in the graphs. A one-way analysis of variance (ANOVA) was used to compare grafted and non-grafted samples, followed by a multiple comparison correction using the Tuckey test. A p-value < 0.05 was deemed significant.

3. Results and Discussion

3.1. Functionalization of titanium surfaces with bioactive polymers

Free radical polymerization of vinyl benzyl phosphonic acid, initiated by the formation of radicals generated by titanium peroxide, was used to covalently graft phosphonate groups to titanium surfaces. The yields of the grafting procedure were computed after the grafting density of poly(VBP) was assessed using the TB colorimetric technique. Controls were titanium oxidized (Ti-OH) and UV-exposed samples. Control samples showed no blue staining, indicating that the coloring was attributable only to the dye being de-complexed from the bioactive polymer's monomer units and, as a result, the proof of poly(VBP) grafted on the titanium surface. However, toluidine blue complexation of the VBP monomer or poly(VBP) adsorbed on the titanium surface might generate the staining. To reject this hypothesis, poly(VBP) and VBP monomers at a concentration of 1 M were introduced with Ti-OH samples for 1 day. The polymerization of VBP monomers was induced by UV irradiation of the VBP aqueous solution containing titanium-oxidized samples, resulting in the formation of radicals of peroxide groups (Fig 1). There was no interaction with the dye beyond this period, confirming that the staining is only due to covalent bonding between the Ti surface and the polymer (results are shown in Fig. 3).
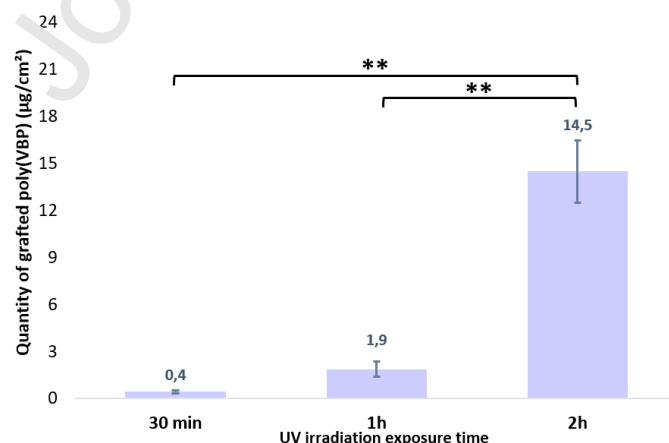

Fig. 2. Quantity of grafted polymers measured as a function of UV irradiation exposure time at a concentration of monomer [VBP] =1 M.

To identify the best grafting conditions, variables including polymerization time (UV irradiation exposure duration) and monomer concentration, were studied.

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The quantity of grafted polymers assessed as a function of UV irradiation exposure duration (Fig. 2) for a monomer concentration of 1 M revealed that the greatest value was attained after 2 hours of UV irradiation exposure (160 mW/cm^2 38% power). Hence, this period was chosen for the investigation of additional characteristics. Furthermore, a concentration of 1 M of VBP monomer allows 14.5±2 μg.cm^{-2} of grafted polymer, whereas a concentration of 0.5 M of VBP monomer produces lower yields (Fig. 3). To achieve high grafting degree yields, all polymerizations were carried out in an inert gas environment with 2 h of UV irradiation and a monomer concentration of 1 M.
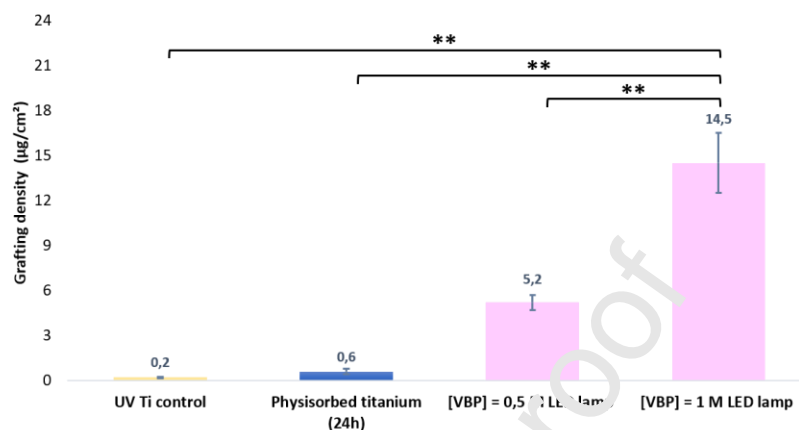

Fig. 3. Amount of grafted polymers measured at two different monomer concentrations.

This study aimed to investigate the potential benefit of a poly(NaSS)-mimicking polymer with a phosphonic acid moiety for improving osseointegration. For this purpose, it was necessary to find the optimal grafting conditions to obtain a good yield.

The exposure time to UV irradiation and the concentration of the monomer were tested to find the best grafting conditions. The colorimetric characterization technique revealed that the grafting of poly(VBP) on titanium surfaces could reach a grafting density of 14.5 μg.cm^{-2}, a high value compared to the one found in the literature for the grafting of bioactive molecules [27-29]. Therefore, the method used in this study is appealing for industrial application since only 2 stages (surface activation then UV irradiation) are required to achieve a high yield of covalent grafting on titanium implants.

3.2. Chemical characterization of grafted titanium surfaces with poly(VBP)

FTIR, XPS, and WCA were used to verify the presence of poly(VBP) on grafted surfaces.

First, FTIR-ATR spectra of grafted titanium revealed the existence of phosphonate groups' distinctive bands: the P=O stretching vibration mode at 1264 cm^{-1}, P-O-C stretching at 985 cm^{-1}, all related to the -PO(CH$_2$) group, and C=C stretching at 1500 cm^{-1} of the phenyl group (Fig. 4). The absorption at 3050 cm^{-1} and 2915 cm^{-1} were attributed to C-H stretching in CH$_2$. The vibrational studies of organic and phosphonate compounds were used to assign these bands [30-33].

The elimination of the signal owing to the C=C stretching vibration mode of the vinyl group of the monomer was the most convincing proof of polymer formation (Fig.4). No specific poly(VBP) peaks were detected on non-grafted Ti surfaces and Ti oxidized samples. The peak observed on non-grafted Ti at 1100 cm^{-1} is attributed to the presence of a thin oxygen layer on the Ti surface. This observation is in agreement with XPS measurements, as shown in Figure 5 and Table 1.
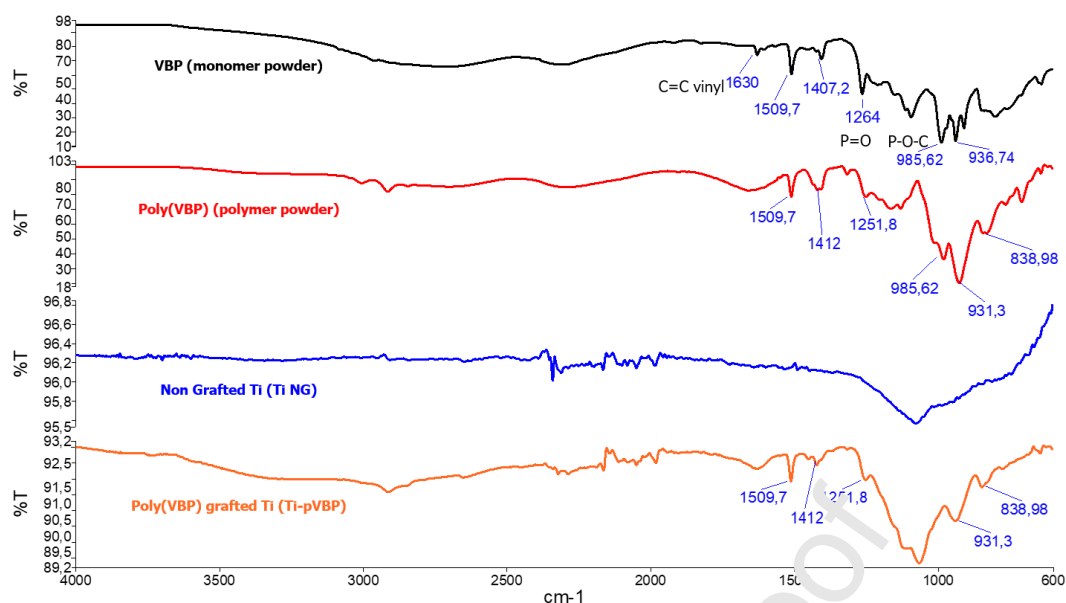
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Fig. 4. FTIR/ATR spectra of VBP monomer, poly(VBP) polymer, non grafted titanium, and poly(VBP) grafted on titanium surface.

XPS studies were performed on materials under various settings: non grafted Ti (Ti NG), oxidized Ti (Ti-OH) and poly(VBP) grafted Ti (Ti-poly(VBP)). Table 1 summarizes the atomic composition determined by XPS measurements. A thin oxide layer of TiO_2 naturally occurs on the non-treated Ti surface, as shown in Figure 5 by the presence of oxygen (O1s) at 530 eV; in addition, adventitious carbonaceous contamination is also observed by the presence of C1s signal at 285 eV. As a result, Ti2p (458 eV), O1s (530 eV), C1s (285 eV) are components classically seen on titanium surfaces [20].

After activation, less carbon contamination was observed at 289.3 eV, Figure 6 (a) on the activated titanium surfaces with a more intense contribution at 285.4 eV. In addition, on the oxygen region, Figure 6 (b), a very intense peak observed at 532.3 eV, assigned to hydroxyls and oxygen radicals, confirmed the activation of the titanium surface.

The titanium surface grafted with poly(VBP) causes a rise in carbon (by 2.17) and an oxygen reduction. The polymer has a high carbon and low oxygen content, which accounts for the observed fluctuation. The primary marker of poly(VBP) is the appearance of the P2p peak at 132 eV, clearly confirming the successful grafting of poly(VBP) at the titanium surface. Moreover, after the poly(VBP) grafting, the titanium substrate is hardly distinguishable, suggesting a polymer equivalent thickness around 10 nm. This conclusion was made by observing the results of the XPS measurements in Table 1. The Ti2p peak (458 eV) decreases with grafting of poly(VBP) (18.7 at. % on ungrafted Ti samples and 0.6 at. % on grafted Ti samples), the titanium substrate becomes hardly visible.

Table 1. Surface composition (at. %) of the various titanium samples as revealed by XPS analysis.

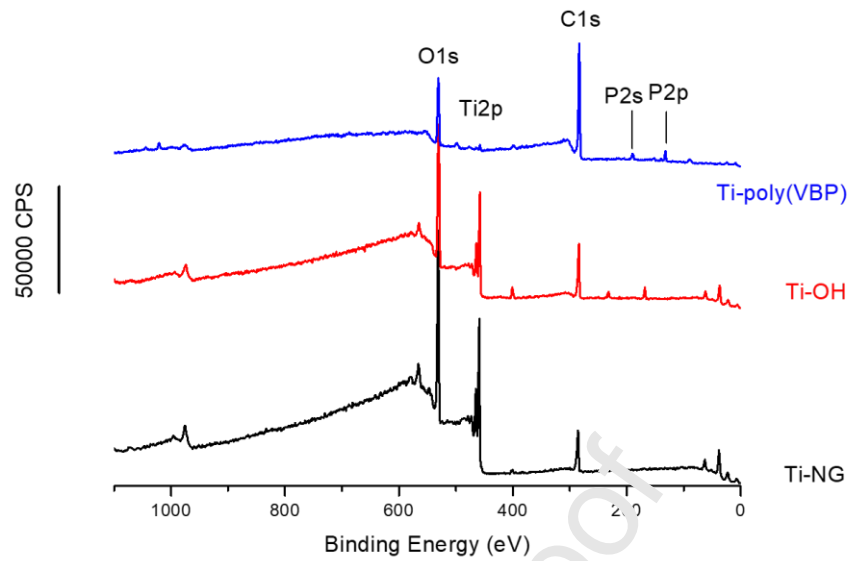| Element	Position (eV)	Ti-NG (atomic %)	Ti-OH (atomic %)	Ti-poly(VBP) (atomic %)
Ti 2p	458	18.7	12.7	0.6
C 1s	285	33.5	42.2	72.7
O 1s	530	47.8	45.1	20.5
P 2p	132	/	/	6.2

Figure 5: XPS survey spectra of titanium surfaces: non grafted (Ti-NG), oxidized (Ti-OH), and grafted with poly(VBP) (Ti-poly(VBP)).
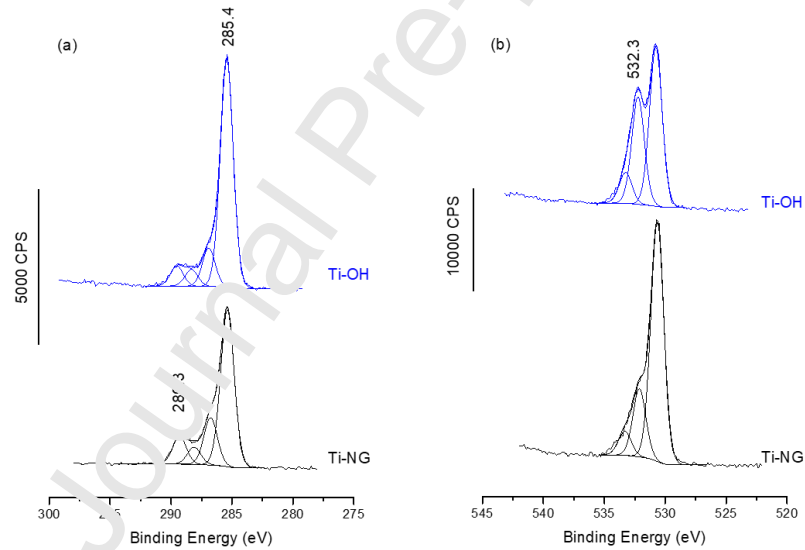

Figure 6: High-Resolution XPS data for (a) C1s and (b) O1s.

To investigate the surface's wettability, WCA was measured on polished Ti, stripped Ti, oxidized Ti, and poly(VBP) grafted Ti (Fig. 7). The hydrophilic nature of the surface is enhanced by the production of Ti-OH (contact angle around 25°) in the first step and the presence of poly(VBP) in the second phase (contact angle around 42°). After the grafting procedure, the Ti surface becomes more hydrophilic compared to a stripped Ti surface with a contact angle of around 66°.
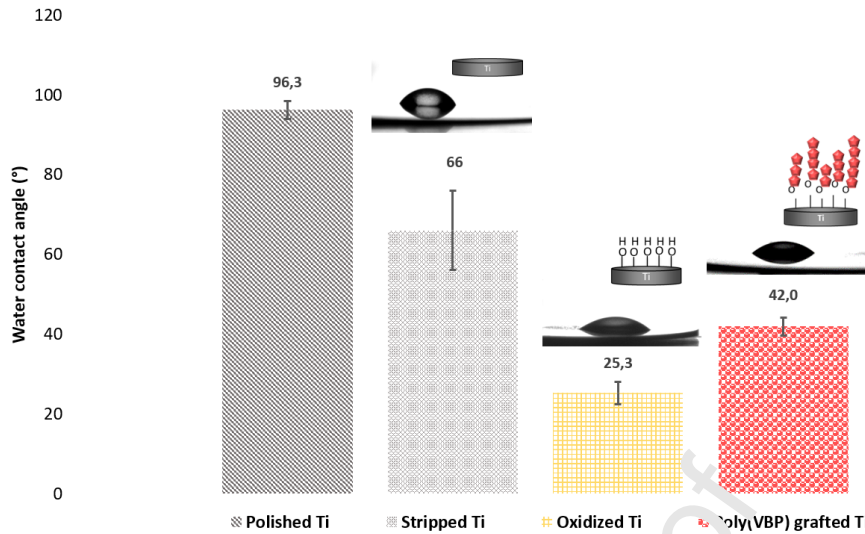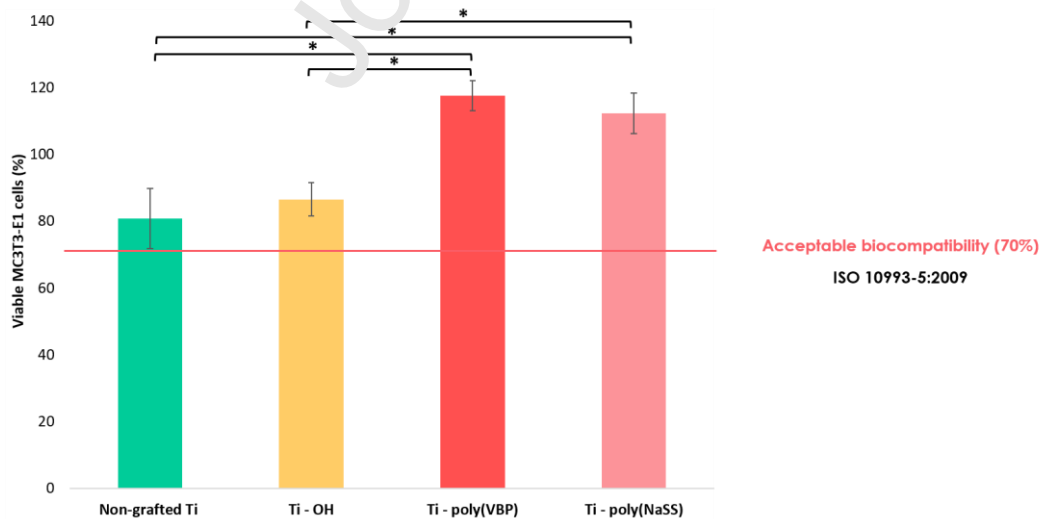
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Fig. 7. Water contact angle measurements on polished Ti, stripped Ti, oxidized and poly(VBP) grafted Ti.

After validating the grafting protocol, the effective presence of the polymer was verified by FTIR-ATR, XPS, and WCA analysis. XPS analysis revealed that the poly(VBP) grafted titanium surfaces have a significant atomic percentage of phosphorus (6.2 at. %). Furthermore, compared to the non-functionalized titanium, the grafted surface becomes more hydrophilic (with a contact angle value that decreases by 20°). This value is consistent with the literature's water contact angle measurements results obtained for phosphonic acid [34]. The hydrophilic/hydrophobic balance of a surface influences cellular spreading at the cells/implant contact, with hydrophilic surfaces having a beneficial influence on cell adherence [35,36].

3.3. Cell viability

The percentage of viable cells was determined by MTT assay after 1 day of MC3T3-E1 cells culture (Fig. 8). All Ti surfaces revealed at least 70% of viable cells during the entire assay and were therefore considered non-cytotoxic. These findings demonstrate that functionalized Ti surfaces do not affect the viable cell rate of MC3T3-E1. Moreover, the value found for poly(VBP) grafted Ti surfaces was substantially (p=0.01) greater than the non-grafted Ti and the oxidized Ti surfaces, indicating a favorable outcome of poly(VBP) on the osteoblast cell response. Furthermore, when comparing poly(NaSS) grafted surfaces (112±6% viable osteoblast cells) and poly(VBP) grafted surfaces (118±4.2% viable osteoblast cells), it is interesting to note a slightly better cellular response for the surfaces grafted with the phosphonic acid-based polymer

Fig. 8. Percentage of viable osteoblast cells over non-grafted titanium, oxidized, poly(VBP), and poly(NaSS) grafted titanium after 24 h incubation.

3.4. Cellular spreading and morphology

Cell spreading and morphology analyses were performed by environmental scanning electron microscopy (SEM) to take a broad look at the morphology of osteoblast cells. MC3T3-E1 osteoblast cells were cultivated on non-grafted Ti, oxidized Ti (Ti-OH), poly(NaSS), and poly(VBP) grafted Ti surfaces during 1 h, 4 h, and 24 h (Fig. 9).

After 1 h of culture, SEM images have shown a considerable variation of the MC3T3-E1 osteoblast cells spreading between Ti surfaces. Osteoblast cells were more confluent on grafted surfaces than on non-grafted surfaces. For the same magnification, the nucleus of the cells was more prominent on the grafted surfaces.

After 4 h of culture, osteoblast cells were connecting with a flattened morphology with lamellipodia extensions on oxidized and grafted surfaces. However, on ungrafted surfaces, the cell expansion was very restrained, with most cells exhibiting a round shape, and no evidence of interconnection between the cells was observed. Osteoblasts cultivated on poly(VBP) grafted surfaces elongate their cytoplasmic across the whole material surface, revealing a solid adhesion capacity of MC3T3-E1 osteoblast to the presence of poly(VBP) onto the surfaces.

After 1 day of culture, osteoblast cells' morphology onto non-grafted surfaces has a more extended and flattened form than the polygonal shape observed on oxidized and grafted surfaces. In addition, it was immediately apparent that the amount of cells was considerably higher on the poly(VBP) grafted surfaces. This observation is consistent with the cell viability experiment result depicted in Fig. 8.
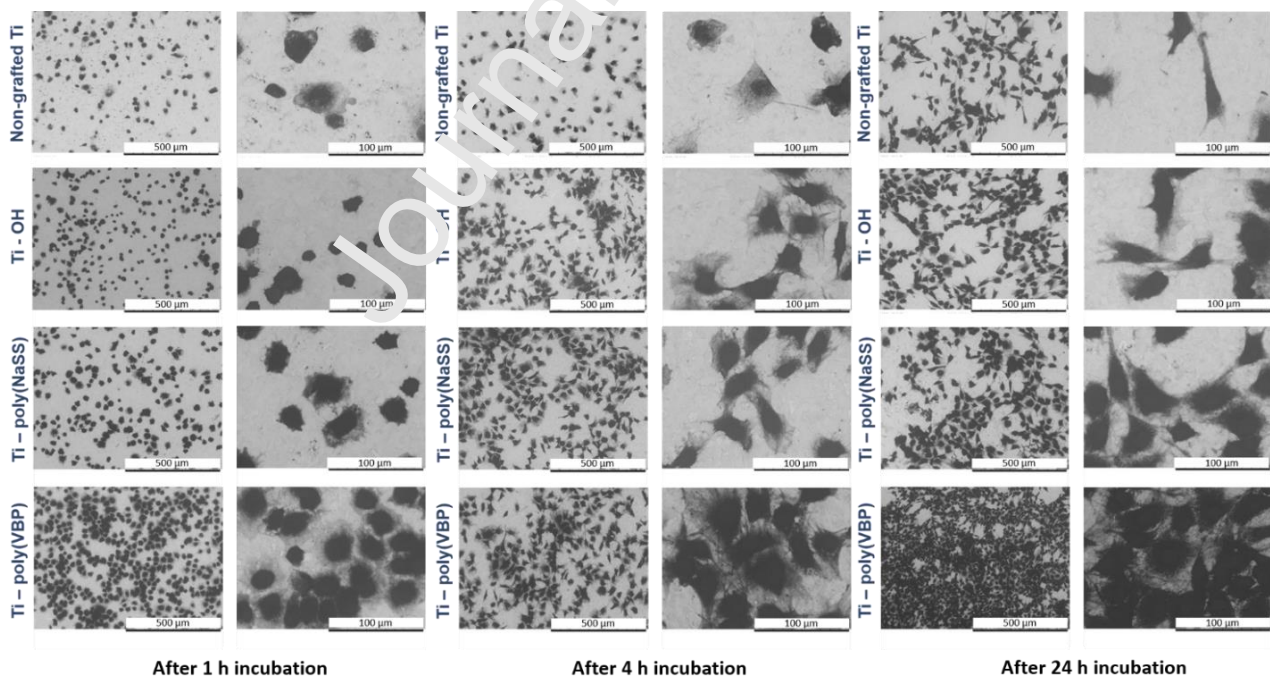

Fig. 9. After 1 h, 4 h, and 24 h incubation, SEM images of osteoblast cells onto the non-grafted, oxidized, poly(NaSS), and poly(VBP) grafted Ti surfaces.

ImageJ software was used to calculate the surface area covered by the cells after 24 h of culture in each condition. This quantification was done on three separate zones of each sample, and it was discovered that the

grafted samples were considerably (p=0.01) more covered than the non-grafted (26 ± 9% surface covered) and oxidized surfaces (29 ± 5%). Furthermore, when compared to surfaces grafted with poly(NaSS) (44 ± 6%), the proportion of osteoblast cells covered on poly(VBP) grafted surfaces (66 ± 4.5%) was more significant (Fig. 10). As a result, adherent cell populations on poly(VBP) grafted surfaces are strikingly improved compared to non-grafted surfaces. The poly(VBP) grafted surfaces promote osteoblast adhesion and spreading, resulting in favorable conditions for future cell growth.
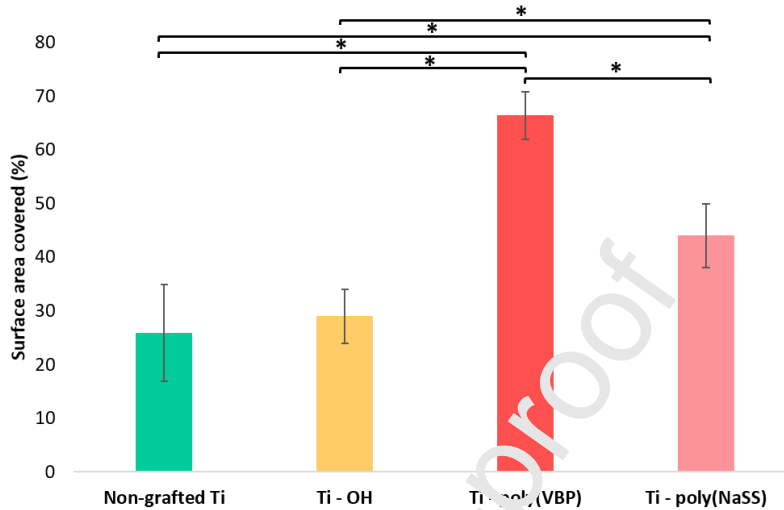

Fig. 10. Mean percentage of non-grafted, oxidized (Ti-OH) poly(VBP) and poly(NaSS) grafted surfaces covered by osteoblast cells after 24 h incubation.

The primary goal of this research was to illustrate the impact of poly(VBP) grafted Ti surfaces on *in vitro* osteogenic properties. In this context, the bioactivity of the chemically modified Ti surfaces grafted with phosphonic acid-based compounds was studied. For this purpose, cell viability, osteoblast cells' morphology, and spreading through SEM and MTT were performed on non-treated titanium and functionalized titanium surfaces. Furthermore, to account for the bioactivity of both bioactive polymers, the grafting of poly(VBP) was compared to the grafting of poly(NaSS). The UV "grafting from" method published by Chouirfa et al [18,26] was used to graft poly(NaSS) onto Ti surfaces. The high values of viable cell densities, improvement of cell morphology, and spreading on poly(VBP) grafted surfaces suggested no cellular toxicity and an enhancement in osteoblast cells' growth. Moreover, after 1 h of culture, SEM images revealed the formation of microcellular extensions called filopodia and adherence of the cells to each other in poly(VBP) grafted Ti substrates. The improved cell spreading could be related to the enhanced hydrophilicity of the surface due to the surface-grafting of phosphonate groups.

3.5. Differentiation and mineralization

ALP is a primary indicator of osteoblast differentiation [25]. The effect of untreated and functionalized Ti surfaces on osteoblast cell development was initially investigated using ALP activity measurements from 7 to 14 days of culture. Results (Fig. 11) showed that ALP concentration was much improved by the grafting of poly(NaSS) and poly(VBP), with maximum concentration found at day 14, which corresponded to the beginning of the extracellular matrix (ECM) development. Before day 14, ALP production was lower because proliferation was dominant. ALP reduced in result of cell maturation's ECM mineralization phase with the apparition of calcium nodules in the second stage of differentiation [37]. For the poly(VBP) grafted surfaces, the ALP concentration started to decrease from day 14, showing the beginning of the mineralization phase. Ti surfaces were therefore evaluated for calcium content after 21 and 28 days of culture (Fig. 11).
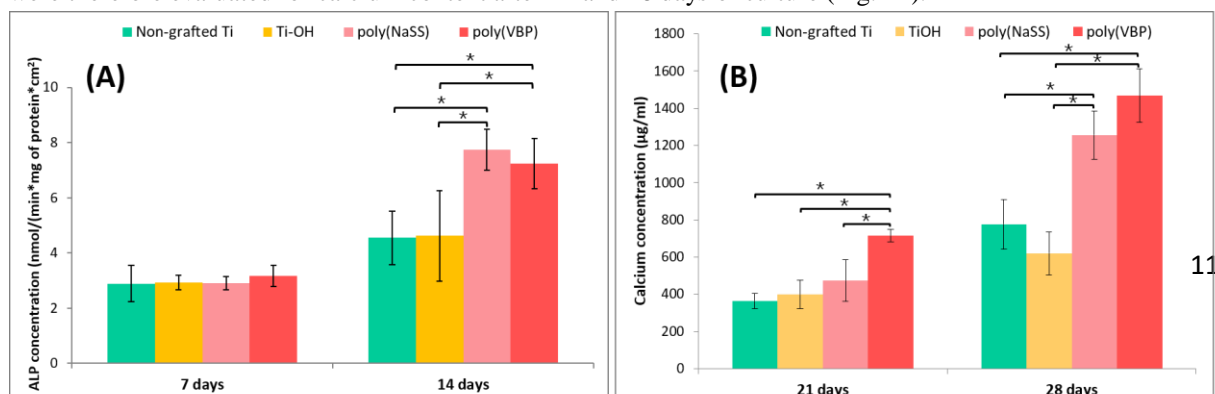

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Fig. 11. ALP activity (A) and ECM mineralization assay (B) of MC3T3-E1 osteoblast cells onto different titanium surfaces (ungrafted, oxidized, poly(NaSS) and poly(VBP) grafted) after respectively 7 and 14, 21, and 28 days of culture.

In accord with ALP results, surfaces grafted with poly(NaSS) and poly(VBP) presented statistically superior mineralization than the non-grafted and oxidized surfaces. The difference was even more pronounced after 4 weeks, indicating that the osteoblasts had matured significantly on the poly(VBP) grafted Ti surfaces. These findings revealed that grafting a titanium sample by poly(VBP) promotes osteoblastic differentiation of MC3T3-E1 cells.

Cell differentiation ability in contact with the implant is crucial for bone regeneration. ALP activity and ECM mineralization were assessed quantitatively to examine osteogenic differentiation of MC3T3-E1 *in vitro*. Poly(VBP) grafting has been demonstrated to promote osteoblast differentiation. Together, these outcomes showed that phosphonate groups grafted on Ti surfaces enhanced osteoblasts' early cell adhesion and spreading processes. In previous investigations, phosphate groups have been shown to promote osteoblast adhesion, spreading, proliferation, and osteogenic differentiation [38-40]. One explanation is that phosphate groups are deprotonated under physiological circumstances, which improves calcium ion chelation, a desirable property for transducing osteogenic promptings [41,42]. As a result, phosphonic acid's strong affinity for calcium ions has prompted biological uses [43].

Furthermore, osteoblast attachment and spreading are affected by adsorbed fibronectin (Fn), and vitronectin (Vn) on biomaterial surfaces [17]. Fn and Vn become more stable in the presence of poly(NaSS), facilitating cell adhesion and growth [44,45]. Although the hypothesis mentioned above partially explains why osteoblast responses are improved on phosphonic acid-based surfaces, follow-up research should be conducted to understand further the protein cells' interaction with poly(VBP).

4. Conclusion

A UV-initiated grafting polymerization technique in a two-step process was successfully developed to graft phosphonate polymers. FTIR-ATR, WCA and XPS data showed that chemically introducing phosphonic acid groups to Ti surfaces was a success. The method developed is easily adaptable to functionalize Ti implants for medical purposes. Moreover, it is possible to extend this process to graft poly(VBP) onto titanium alloys, which are extensively encountered in biomedical equipment. Cell survival, ALP activity, and calcification of MC3T3-E1 osteoblast cells cultured onto the poly(VBP) grafted titanium surfaces revealed increased differentiation of these cells, implying the effect of improved grafted implant osseointegration response. The Ti–metal surface's covalently bonded with phosphonic acid molecules act as a scaffold for new bone growth, allowing the implant to attach to the host tissue. These results indicate that poly(VBP) grafted Ti implants could be used in biological applications.

The next stage of our research will further understand the mechanisms at the atomic and molecular levels involved in cell-protein interaction on grafted titanium and perform in vivo study in a rabbit model.

CRediT authorship contribution statement

Caroline Pereira: Methodology, Investigation, Resources, Validation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Jean-Sébastien Baumann:** Resources. **Vincent Humblot:** Resources, Data curation, Formal analysis, Validation, Writing – review & editing. **Céline Falentin-Daudré:** Supervision, Conceptualization, Methodology, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Validation, Writing – review & editing.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CRediT authorship contribution statement

Caroline Pereira: Methodology, Investigation, Resources, Validation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Jean-Sébastien Baumann:** Resources. **Vincent Humblot:** Resources, Data curation, Formal analysis, Validation, Writing – review & editing. **Céline Falentin-Daudré:** Supervision, Conceptualization, Methodology, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Validation, Writing – review & editing.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

References

[1] P.-I. Brånemark, U. Breine, R. Adell, B.O. Hansson, J. Lindström, Å. Ohlsson, Intra-Osseous Anchorage of Dental Prostheses:I. Experimental Studies 3 (1969) 81–100. https://doi.org/10.3109/02844316909036699.

[2] M. Esposito, J.-M. Hirsch, U. Lekholm, P. Thomsen, Biological factors contributing to failures of osseointegrated oral implants, European Journal of Oral Sciences. 106 (1998) 527–551. https://doi.org/10.1046/j.0909-8836..t01.2-.x.

[3] A.M. Roos-Jansaker, S. Renvert, J. Egelberg, Treatment of peri-implant infections: a literature review, J. Clin. Periodontol. 30 (2003) 467–485. https://doi.org/10.1034/j.1600-051X.2003.00296.x.

[4] J.E. Ellingsen, Surface configurations of dental implants, Periodontol 2000. 17 (1998) 36–46. https://doi.org/10.1111/j.1600-0757.1998.tb00121.x.

[5] Y.-J. Park, H.-J. Song, I. Kim, H.-S. Yang, Surface characteristics and bioactivity of oxide film on titanium metal formed by thermal oxidation, J Mater Sci: Mater Med. 18 (2007) 565–575. https://doi.org/10.1007/s10856-007-2303-7.

[6] L. Saldaña, V. Barranco, J.L. González-Carrasco, M. Rodríguez, L. Munuera, N. Vilaboa, Thermal oxidation enhances early interactions between human osteoblasts and alumina blasted Ti6Al4V alloy, J. Biomed. Mater. Res. 81A (2007) 334–346. https://doi.org/10.1002/jbm.a.30994.

[7] A. Carradó, Structural, Microstructural, and Residual Stress Investigations of Plasma-Sprayed Hydroxyapatite on Ti-6Al-4 V, ACS Appl. Mater. Interfaces. 2 (2010) 561–565. https://doi.org/10.1021/am900763j.

[8] Y. Liu, P. Layrolle, J. de Bruijn, C. van Blitterswijk, K. de Groot, Biomimetic coprecipitation of calcium phosphate and bovine serum albumin on titanium alloy, J. Biomed. Mater. Res. 57 (2001) 327–335. https://doi.org/10.1002/1097-4636(20011205)57:3<327::AID-JBM1175>3.0.CO;2-J.

[9] Y.-T. Sul, C.B. Johansson, S. Petronis, A. Krozer, A. Wennerberg, T. Albrektsson, Characteristics of the surface oxides on turned and electrochemically oxidized pure titanium implants up to dielectric breakdown:

the oxide thickness, micropore configurations, surface roughness, crystal structure and chemical composition, Biomaterials 23 (2002) 491-501. https://doi.org/10.1016/S0142-9612(01)00131-4.

[10] L. Le Guéhennec, A. Soueidan, P. Layrolle, Y. Amouriq, Surface treatments of titanium dental implants for rapid osseointegration, Dental Materials. 23 (2007) 844–854. https://doi.org/10.1016/j.dental.2006.06.025.

[11] Y.-J. Park, H.-J. Song, I. Kim, H.-S. Yang, Surface characteristics and bioactivity of oxide film on titanium metal formed by thermal oxidation, J Mater Sci: Mater Med. 18 (2007) 565–575. https://doi.org/10.1007/s10856-007-2303-7.

[12] A. Michiardi, G. Hélary, P.-C.T. Nguyen, L.J. Gamble, F. Anagnostou, D.G. Castner, V. Migonney, Bioactive polymer grafting onto titanium alloy surfaces, Acta Biomaterialia. 6 (2010) 667–675. https://doi.org/10.1016/j.actbio.2009.08.043.

[13] H. Chouirfa, M.D.M. Evans, D.G. Castner, Grafting of architecture controlled poly(styrene sodium sulfonate) onto titanium surfaces using bio-adhesive molecules: Surface characterization and biological properties, Biointerphases 12, 02C418 (2017). http://dx.doi.org/10.1116/1.4985608.

[14] N. Adden, L.J. Gamble, D.G. Castner, A. Hoffmann, G. Gross, H. Menzel, Phosphonic Acid Monolayers for Binding of Bioactive Molecules to Titanium Surfaces, Langmuir. 22 (2006) 8197–8204. https://doi.org/10.1021/la060754c.

[15] F. El Khadali, G. Hélary, G. Pavon-Djavid, V. Migonney, Modulating Fibroblast Cell Proliferation with Functionalized Poly(methyl methacrylate) Based Copolymers: Chemical Composition and Monomer Distribution Effect, Biomacromolecules. 3 (2002) 51–56. https://doi.org/10.1021/bm015563x.

[16] F. Anagnostou, A. Debet, G. Pavon-Djavid, Z. Goudaby, G. Hélary, V. Migonney, Osteoblast functions on functionalized PMMA-based polymers exhibiting Staphylococcus aureus adhesion inhibition, Biomaterials. 27 (2006) 3912–3919. https://doi.org/10.1016/j.biomaterials.2006.03.004.

[17] H.P. Felgueiras, M.D.M. Evans, V. Migonney, Contribution of fibronectin and vitronectin to the adhesion and morphology of MC3T3-E1 osteoblastic cells to poly(NaSS) grafted Ti6Al4V, Acta Biomaterialia. 28 (2015) 225–233. https://doi.org/10.1016/j.actbio.2015.09.030.

[18] H. Chouirfa, V. Migonney, C. Falentin-Daudré, Grafting bioactive polymers onto titanium implants by UV irradiation, RSC Adv. 6 (2016) 13766–13771. https://doi.org/10.1039/C5RA24497H.

[19] P. Datta, J. Chatterjee, S. Dhara, Electrospun nanofibers of a phosphorylated polymer—A bioinspired approach for bone graft applications, Colloids and Surfaces B: Biointerfaces. 94 (2012) 177–183. https://doi.org/10.1016/j.colsurfb.2012.01.033.

[20] C. Viornery, Y. Chevolot, D. Léonard, B.-O. Aronsson, P. Péchy, H.J. Mathieu, P. Descouts, M. Grätzel, Surface Modification of Titanium with Phosphonic Acid To Improve Bone Bonding: Characterization by XPS and ToF-SIMS, Langmuir. 18 (2002) 2582–2589. https://doi.org/10.1021/la010908i.

[21] C. Viornery, H.L. Guenther, B.-O. Aronsson, P. Péchy, P. Descouts, M. Grätzel, Osteoblast culture on polished titanium disks modified with phosphonic acids: Osteoblast Culture on Polished Titanium, J. Biomed. Mater. Res. 62 (2002) 149–155. https://doi.org/10.1002/jbm.10205.

[22] R.E. Dey, I. Wimpenny, J.E. Gough, D.C. Watts, P.M. Budd, Poly(vinylphosphonic acid- co -acrylic acid) hydrogels: The effect of copolymer composition on osteoblast adhesion and proliferation: PVPA- co -AA HYDROGELS, J. Biomed. Mater. Res. 106 (2018) 255–264. https://doi.org/10.1002/jbm.a.36234.

[23] R.E. Dey, X. Zhong, P.J. Youle, Q.G. Wang, I. Wimpenny, S. Downes, J.A. Hoyland, D.C. Watts, J.E. Gough, P.M. Budd, Synthesis and Characterization of Poly(vinylphosphonic acid- co -acrylic acid) Copolymers for Application in Bone Tissue Scaffolds, Macromolecules. 49 (2016) 2656–2662. https://doi.org/10.1021/acs.macromol.5b02594.

[24] P.M. López-Pérez, R.M.P. da Silva, R.A. Sousa, I. Pashkuleva, R.L. Reis, Plasma-induced polymerization as a tool for surface functionalization of polymer scaffolds for bone tissue engineering: An in vitro study, Acta Biomaterialia. 6 (2010) 3704–3712. https://doi.org/10.1016/j.actbio.2010.03.008.

[25] G. Helary, F. Noirclere, J. Mayingi, V. Migonney, A new approach to graft bioactive polymer on titanium implants: Improvement of MG 63 cell differentiation onto this coating, Acta Biomaterialia. 5 (2009) 124–133. https://doi.org/10.1016/j.actbio.2008.07.037.

[26] C. Falentin-Daudré, V. Migonney, H. Chouirfa, and J. S. Baumann, WO patent PCT/EP2016/068909 (August 7th, 2015).

[27] M.C. Porté-Durrieu, F. Guillemot, S. Pallu, C. Labrugère, B. Brouillaud, R. Bareille, J. Amédée, N. Barthe, M. Dard, C. Baquey, Cyclo-(DfKRG) peptide grafting onto Ti–6Al–4V: physical characterization and interest towards human osteoprogenitor cells adhesion, Biomaterials. 25 (2004) 4837–4846. https://doi.org/10.1016/j.biomaterials.2003.11.037.

[28] R. Müller, J. Abke, E. Schnell, D. Scharnweber, R. Kujat, C. Englert, D. Taheri, M. Nerlich, P. Angele, Influence of surface pretreatment of titanium- and cobalt-based biomaterials on covalent immobilization of fibrillar collagen, Biomaterials. 27 (2006) 4059–4068. https://doi.org/10.1016/j.biomaterials.2006.03.019.

[29] C. Chollet, C. Chanseau, B. Brouillaud, M.C. Durrieu, RGD peptides grafting onto poly(ethylene terephthalate) with well controlled densities, Biomolecular Engineering. 24 (2007) 477–482. https://doi.org/10.1016/j.bioeng.2007.07.012.

[30] Nakanishi, K. Infrared absorption spectroscopy (Holden-Day, San Francisco, 1962).

[31] M.J. Danilich, D.J. Burton, R.E. Marchant, Infrared study of perfluorovinylphosphonic acid, perfluoroallylphosphonic acid, and pentafluoroallyldiethylphosphonate, Vibrational Spectroscopy. 9 (1995) 229–234. https://doi.org/10.1016/0924-2031(95)00018-P.

[32] A. Popa, C.-M. Davidescu, P. Negrea, G. Ilia, A. Katsaros, K.D. Demadis, Synthesis and Characterization of Phosphonate Ester/Phosphonic Acid Grafted Styrene−Divinylbenzene Copolymer Microbeads and Their Utility in Adsorption of Divalent Metal Ions in Aqueous Solutions, Ind. Eng. Chem. Res. 47 (2008) 2010–2017. https://doi.org/10.1021/ie0708802.

[33] M. Yamabe, K. Akiyama, Y. Akatsuka, M. Kato, Novel phosphonated perfluorocarbon polymers, European Polymer Journal. (2000) 7. https://doi.org/10.1016/S0014-3057(99)00158-5.

[34] M.P. Danahy, M.J. Avaltroni, K.S. Midwood, J.E. Schwarzbauer, J. Schwartz, Self-assembled Monolayers of α,ω-Diphosphonic Acids on Ti Enable Complete or Spatially Controlled Surface Derivatization, Langmuir. 20 (2004) 5333–5337. https://doi.org/10.1021/la036084h.

[35] B. Feng, J.Y. Chen, S.K. Qi, L. He, J.Z. Zhao, X.D. Zhang, Characterization of surface oxide films on titanium and bioactivity, Journal of Materials Science: Materials in Medicine. 13 (2002) 457–464. https://doi.org/10.1023/A:1014737831371.

[36] G. Zhao, Z. Schwartz, M. Wieland, F. Rupp, J. Geis-Gerstorfer, D.L. Cochran, B.D. Boyan, High surface energy enhances cell response to titanium substrate microstructure, J. Biomed. Mater. Res. 74A (2005) 49–58. https://doi.org/10.1002/jbm.a.30320.

[37] J.Y. Martin, D.D. Dean, D.L. Cochran, J. Simson, B.D. Boyan, Z. Schwartz, Proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63) cultured on previously used titanium surfaces, Clin. Oral Implants Res. 7 (1996) 27–37.

[38] D.M. Zheng, K.G. Neoh, E.-T. Kang, Immobilization of alendronate on titanium via its different functional groups and the subsequent effects on cell functions., Journal of Colloid and Interface Science. 487 (2017) 1–11. https://doi.org/10.1016/j.jcis.2016.10.014.

[39] M. Dadsetan, M. Giuliani, F. Wanivenhaus, M.B. Runge, J.E. Charlesworth, M.J. Yaszemski, Incorporation of phosphate group modulates bone cell attachment and differentiation on oligo(polyethylene glycol) fumarate hydrogel, Acta Biomaterialia. 8 (2012) 1430–1439. https://doi.org/10.1016/j.actbio.2011.12.031.

[40] R. Nakaoka, Y. Yamakoshi, K. Isama, T. Tsuchiya, Effects of surface chemistry prepared by self-assembled monolayers on osteoblast behavior., Journal of Biomedical Materials Research. Part A. 94 2 (2010) 524–32.

[41] A.-S. Wagner, K. Glenske, V. Wolf, D. Fietz, S. Mazurek, T. Hanke, A. Moritz, S. Arnhold, S. Wenisch, Osteogenic differentiation capacity of human mesenchymal stromal cells in response to extracellular calcium with special regard to connexin 43, Annals of Anatomy - Anatomischer Anzeiger. 209 (2017) 18–24. https://doi.org/10.1016/j.aanat.2016.09.005.

[42] L.F. Mellor, M. Mohiti-Asli, J. Williams, A. Kannan, M.R. Dent, F. Guilak, E.G. Loboa, Extracellular Calcium Modulates Chondrogenic and Osteogenic Differentiation of Human Adipose-Derived Stem Cells: A Novel Approach for Osteochondral Tissue Engineering Using Single Stem Cell Source, Tissue Engineering. Part A. 21 (2015) 2323—2333. https://doi.org/10.1089/ten.tea.2014.0572.

[43] C.M. Sevrain, M. Berchel, H. Couthon, P.-A. Jaffrès, Phosphonic acid: preparation and applications, Beilstein J. Org. Chem. 13 (2017) 2186–2213. https://doi.org/10.3762/bjoc.13.219.

[44] D.F. Mosher, L.T. Furcht, Fibronectin: Review of its Structure and Possible Functions, Journal of Investigative Dermatology. 77 (1981) 175–180. https://doi.org/10.1111/1523-1747.ep12479791.

[45] J.R. Potts, I.D. Campbell, Structure and function of fibronectin modules, Matrix Biology. 15 (1996) 313–320. https://doi.org/10.1016/S0945-053X(96)90133-X.

Biological effect of direct grafting by ultraviolet irradiation of vinyl benzyl phosphonic acid onto titanium surfaces

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Highlights:

- An ultraviolet-initiated grafting polymerization of phosphonic acid is developed.

- Surface and biological properties of functionalized titanium are assessed.

- Improvement of osteoblast cells adhesion and spreading processes are presented.

- Enhanced functionalization of titanium surfaces for biomedical applications.

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